

URATE OXIDASE

The present application claims benefit of U.S. Provisional Application No. 60/095,489, filed August 6, 1998, the entire contents of which is incorporated herein by reference.

5 The invention disclosed herein was made with U.S. Government support under Grant No. DK48529, awarded by the National Institutes of Health. The Government has certain rights in the invention.

10 The present invention relates, in general, to urate oxidase (uricase) proteins and nucleic acid molecules encoding same. In particular, the invention relates to uricase proteins which are particularly useful as, for example, intermediates for making improved modified uricase proteins with reduced immunogenicity and increased bioavailability. The preferred modified uricase proteins of the present invention include the uricase proteins covalently bound to poly(ethylene glycols) or poly(ethylene oxides). The present invention provides, therefore, uricase proteins, antibodies which
15 specifically bind with the proteins, nucleic acid molecules encoding the uricase proteins and useful fragments thereof, vectors containing the nucleic acid molecules, host cells containing the vectors and methods of using and making the uricase proteins and nucleic acid molecules.

Background

20 Gout is the most common inflammatory joint disease in men over age 40 (Roubenoff 1990). Painful gouty arthritis occurs when an elevated blood level of uric acid (*hyperuricemia*) leads to the episodic formation of microscopic crystals of monosodium urate monohydrate in joints. Over time, chronic hyperuricemia can also result in destructive crystalline urate deposits (*tophi*) around joints, in soft tissues, and
25 in some organs (Hershfield 1996). Uric acid has limited solubility in urine and when overexcreted (*hyperuricosuria*) can cause kidney stones (*uricolithiasis*). In patients with certain malignancies, particularly leukemia and lymphoma, marked hyperuricemia and hyperuricosuria (due to enhanced tumor cell turnover and lysis during chemotherapy) pose a serious risk of acute, obstructive renal failure (Sandberg et al. 1956; Gold and
30 Fritz 1957; Cohen et al. 1980; Jones et al. 1990). Severe hyperuricemia and gout are associated with renal dysfunction from various causes, including cyclosporine therapy to

prevent organ allograft rejection (West et al. 1987; Venkateshan et al. 1990; Ahn et al. 1992; Delaney et al. 1992; George and Mandell 1995).

Hyperuricemia can result from both urate overproduction and underexcretion (Hershfield and Seegmiller 1976; Kelley et al. 1989; Becker and Roessler 1995). When mild, hyperuricemia can be controlled with diet, but when pronounced and associated with serious clinical consequences, it requires treatment with drugs, either a uricosuric agent that promotes uric acid excretion (ineffective if renal function is reduced), or the xanthine oxidase inhibitor allopurinol, which blocks urate formation. Allopurinol is the mainstay of therapy in patients with tophaceous gout, renal insufficiency, leukemia, and some inherited disorders. Treatment for hyperuricemia is generally effective and well-tolerated. However, some patients with disfiguring, incapacitating tophaceous gout are refractory to all conventional therapy (Becker 1988; Fam 1990; Rosenthal and Ryan 1995). Moreover, ~2% of patients treated with allopurinol develop allergic reactions, and a severe hypersensitivity syndrome occurs in ~0.4% (Singer and Wallace 1986; Arellano and Sacristan 1993). This often life-threatening syndrome can cause acute renal and hepatic failure, and severe skin injury (toxic epidermal necrolysis, exfoliative dermatitis, erythema multiforme, Stevens-Johnson syndrome). Allopurinol also interferes with the metabolism of azathioprine and 6-mercaptopurine, drugs used in the treatment of leukemia and for prevention of organ allograft rejection, conditions in which marked hyperuricemia occurs and may cause severe gout or threaten renal function.

Ultimately, hyperuricemia is the result of mutational inactivation of the human gene for urate oxidase (uricase) during evolution (Wu et al. 1989; Wu et al. 1992).

Active uricase in liver peroxisomes of most non-human primates and other mammals converts urate to allantoin (+ CO₂ and H₂O₂), which is 80-100 times more soluble than uric acid and is handled more efficiently by the kidney. Parenteral uricase, prepared from *Aspergillus flavus* (Uricozyme[®], Clin-Midy, Paris), has been used to treat severe hyperuricemia associated with leukemia chemotherapy for over 20 years in France and Italy (London and Hudson 1957; Kissel et al. 1968; Brogard et al. 1972; Kissel et al. 1972; Potaux et al. 1975; Zittoun et al. 1976; Brogard et al. 1978; Masera et al. 1982), and has been used in recent clinical trials in leukemia patients in the US (Pui et al.

1997). Uricase has a more rapid onset of action than allopurinol (Masera et al. 1982; Pui et al. 1997). In patients with gout, uricase infusions can interrupt acute attacks and decrease the size of tophi (Kissel et al. 1968; Potaux et al. 1975; Brogard et al. 1978).

Though effective for treating acute hyperuricemia during a short course of chemotherapy, daily infusion of *A. flavus* uricase would be a serious drawback for treating recurrent or tophaceous gout. In addition, efficacy of *A. flavus* uricase diminishes quickly in patients who develop anti-uricase antibodies (Kissel et al. 1968; Brogard et al. 1978; Escudier et al. 1984; Mourad et al. 1984; Sibony et al. 1984). Serious allergic reactions, including anaphylaxis, have occurred (Donadio et al. 1981; Montagnac and Schillinger 1990; Pui et al. 1997). A longer-acting, less immunogenic preparation of uricase is clearly needed for chronic therapy.

One approach for sequestering exogenous enzymes from proteases and the immune system involves covalent attachment of the inert, nontoxic polymer, monomethoxypolyethylene glycol (PEG) to the surface of proteins (Harris and Zalipsky 1997). Use of PEGs with Mr ~1,000 to >10,000 was first shown to prolong the circulating life and reduce the immunogenicity of several foreign proteins in animals (Abuchowski et al. 1977a; Abuchowski et al. 1977b; Davis et al. 1981a; Abuchowski et al. 1984; Davis et al. 1991). In 1990, bovine adenosine deaminase (ADA) modified with PEG of Mr 5000 (PEG-ADA, ADAGEN[®], produced by Enzon, Inc.) became the first PEGylated protein to be approved by the United States Food and Drug Administration, for treatment of severe combined immune deficiency disease due to ADA deficiency (Hershfield et al. 1987). Experience over the past 12 years has shown that anti-ADA antibodies can be detected by a sensitive ELISA in most patients during chronic treatment with PEG-ADA, but there have been no allergic or hypersensitivity reactions; accelerated clearance of PEG-ADA has occurred in a few anti-ADA antibody producing patients, but this has usually been a transient effect (Chaffee et al. 1992; Hershfield 1997). It should be appreciated that immune function of patients with ADA deficiency usually does not become normal during treatment with PEG-ADA (Hershfield 1995; Hershfield and Mitchell 1995). Thus, immunogenicity might be a more significant problem in developing a PEGylated enzyme for chronic treatment of patients with normal immune function.

Immunogenicity will be understood by one of ordinary skill as relating to the induction of an immune response by an injected preparation of an antigen (such as PEG-modified protein or unmodified protein), while antigenicity refers to the reaction of an antigen with preexisting antibodies. Collectively, antigenicity and immunogenicity are referred to as immunoreactivity. In previous studies of PEG-uricase, immunoreactivity was assessed by a variety of methods, including: the reaction *in vitro* of PEG-uricase with preformed antibodies; measurements of induced antibody synthesis; and accelerated clearance rates after repeated injections.

PEGylation has been shown to reduce the immunogenicity and prolong the circulating life of fungal and porcine uricases in animals (Chen et al. 1981; Savoca et al. 1984; Tsuji et al. 1985; Veronese et al. 1997). PEG-modified *Candida* uricase rapidly lowered serum urate to undetectable levels in 5 normouricemic human volunteers (Davis et al. 1981b). PEGylated *Arthrobacter* uricase produced by Enzon, Inc. was used on a compassionate basis to treat an allopurinol-hypersensitive patient with lymphoma, who presented with renal failure and marked hyperuricemia (Chua et al. 1988; Greenberg and Hershfield 1989). Four intramuscular injections were administered over about two weeks. During this brief period, hyperuricemia was controlled and no anti-uricase antibody could be detected by ELISA in the patient's plasma. Further use and clinical development of this preparation has not been pursued.

To date, no form of uricase or PEG-uricase has been developed that has a suitably long circulating life and sufficiently reduced immunogenicity for safe and reliable use in chronic therapy. The aim of this invention is to provide an improved form of uricase that, in combination with PEGylation, can meet these requirements. The invention is a unique recombinant uricase of mammalian derivation, which has been modified by mutation in a manner that has been shown to enhance the ability of PEGylation to mask potentially immunogenic epitopes.

Summary of the Invention

It is a general object of the present invention to provide novel uricase proteins and nucleic acid sequences encoding same.

It is another object of the present invention to provide a method of purifying recombinantly produced uricase proteins, such as those described herein.

It is a further object of the present invention to provide a method of reducing the amount of uric acid in a body fluid of a mammal by administering a composition containing a uricase protein of the present invention to the mammal.

It is yet another object of the present invention to provide antibodies to the uricase proteins described herein.

It is another object of the present invention to provide vectors and host cells containing the nucleic acid sequences described herein and methods of using same to produce the uricase proteins coded by same.

The present invention provides uricase proteins which may be used to produce a substantially non-immunogenic PEG-uricase that retains all or nearly all of the uricolytic activity of the unmodified enzyme. Uricolytic activity is expressed herein in International Units (IU) per mg protein wherein an IU of uricase activity is defined as the amount of enzyme which consumes one micromole of uric acid per minute.

The present invention provides a recombinant uricase protein of a mammalian species which has been modified to insert one or more lysine residues. Recombinant protein, as used herein, refers to any artificially produced protein and is distinguished from naturally produced proteins (i.e., that are produced in tissues of an animal that possesses only the natural gene for the specific protein of interest). Protein includes peptides and amino acid sequences. The recombinant uricase protein of the present invention may be a chimera or hybrid of two or more mammalian proteins, peptides or amino acid sequences. In one embodiment, the present invention can be used to prepare a recombinant uricase protein of a mammalian species, which protein has been modified to increase the number of lysines to the point where, after PEGylation of the recombinant uricase protein, the PEGylated uricase product is substantially as enzymatically active as the unmodified uricase and the PEGylated uricase product is not unacceptably immunogenic. Truncated forms of the uricases of the present invention are also contemplated wherein amino and/or carboxy terminal ends of the uricase may not be present. Preferably, the uricase is not truncated to the extent that lysines are removed.

One of ordinary skill will appreciate that the conjugated uricase-carrier complex must not contain so many linkages as to substantially reduce the enzymatic activity of the uricase or too few linkages so as to remain unacceptably immunogenic. Preferably, the conjugate will retain at least about 70% to about 90% of the uricolytic activity of the unmodified uricase protein while being more stable, such that it retains its enzymatic activity on storage, in mammalian plasma and/or serum at physiological temperature, as compared to the unmodified uricase protein. Retention of at least about 80% to about 85% of the uricolytic activity would be acceptable. Moreover, in a preferred embodiment, the conjugate provides a substantially reduced immunogenicity and/or immunoreactivity than the unmodified uricase protein. In one embodiment, the present invention provides a uricase protein described herein which can be modified by attachment to a non-toxic, non-immunogenic, pharmaceutically acceptable carrier, such as PEG, by covalent linkage to at least 1 of the lysines contained in the uricase protein. Alternatively, the uricase protein is modified by covalent attachment to a carrier through less than about 10 lysines of its amino acid sequence. Attachment to any of 2, 3, 4, 5, 6, 7, 8, or 9 of the lysines are contemplated as alternative embodiments.

The uricase protein of the present invention is a recombinant molecule which includes segments of porcine and baboon liver uricase proteins. A modified baboon sequence is also provided. In one embodiment, the present invention provides a chimeric pig-baboon uricase (PBC uricase (SEQ ID NO:2)) which includes amino acids (aa) 1-225 of porcine uricase (SEQ ID NO:7) and aa 226-304 of baboon uricase (SEQ ID NO:6) (see also sequence in Figure 5). In another embodiment, the present invention provides a chimeric pig-baboon uricase (PKS uricase) which includes aa 1-288 of porcine uricase and aa 289-304 of baboon uricase (SEQ ID NO: 4). Truncated derivatives of PBC and PKS are also contemplated. Preferred truncated forms are PBC and PKS proteins truncated to delete either the 6 amino terminal amino acids or the 3 carboxy terminal amino acids, or both. Representative sequences are given in SEQ ID NO:s 8 (PBC amino truncated), 9 (PBC carboxy truncated), 10 (PKS amino truncated) and 11 (PKS carboxy truncated). Each of the PBC uricase, PKS uricase and their truncated forms have one to four more lysines than are found in other mammalian uricases that have been cloned.

The present invention provides nucleic acid (DNA and RNA) molecules (sequences), including isolated, purified and/or cloned forms of the nucleic acid molecules, which code for the uricase proteins and truncated proteins described herein. Preferred embodiments are shown in SEQ ID NO:1 (PBC uricase) and SEQ ID NO:3 (PKS uricase).

Vectors (expression and cloning) including these nucleic acid molecules are also provided by the present invention.

Moreover, the present invention provides host cells containing these vectors.

Antibodies which specifically bind to the uricase proteins of the present invention are also provided. Antibodies to the amino portion to the pig uricase and antibodies to the carboxy portion of baboon uricase, when used in conjunction, should be useful in detecting PBC, or other similar chimeric proteins. Preferably, the antibody to the amino portion of the chimeric uricase should not recognize the amino portion of the baboon uricase and similarly, the antibody to the carboxy portion of the chimeric uricase should not recognize the carboxy portion of the pig uricase. More preferably, antibodies are provided which specifically bind PBC or PKS but do not bind the native proteins, such as pig and/or baboon uricases.

In another embodiment, the present invention can be used to prepare a pharmaceutical composition for reducing the amount of uric acid in body fluids, such as urine and/or serum or plasma, containing at least one of the uricase proteins or uricase conjugates described herein and a pharmaceutically acceptable carrier, diluent or excipient.

The present invention also may be used in a method for reducing the amount of uric acid in body fluids of a mammal. The method includes administering to a mammal an uric acid-lowering effective amount of a composition containing a uricase protein or uricase conjugate of the present invention and a diluent, carrier or excipient, which is preferably a pharmaceutically acceptable carrier, diluent or excipient. The mammal to be treated is preferably a human.

The administering step may be, for example, injection by intravenous, intradermal, subcutaneous, intramuscular or intraperitoneal routes. The elevated uric acid levels may be in blood or urine, and may be associated with gout, tophi, renal insufficiency, organ transplantation or malignant disease.

In another embodiment, the present invention provides a method for isolating and or purifying a uricase from a solution of uricase containing, for example, cellular and subcellular debris from, for example, a recombinant production process. Preferably, the method of purification takes advantage of the limited solubility of mammalian uricase at low pH (Conley et al. 1979), by washing the crude recombinant extract at a pH of about 7 to about 8.5 to remove a majority of the proteins that are soluble at this low pH range, whereafter active uricase is solubilized in a buffer, preferably sodium carbonate buffer, at a pH of about 10-11, preferably about 10.2. The solubilized active uricase may then be applied to an anion exchange column, such as a Q Sepharose column, which is washed with low to high salt gradient in a buffer at a pH of about 8.5, after which purified uricase is obtained by eluting with a sodium chloride gradient in sodium carbonate buffer at a pH of about 10 to about 11, preferably about 10.2. The enzyme may be further purified by gel filtration chromatography at a pH of about 10 to about 11. At this stage, the enzyme may be further purified by lowering the pH to about 8.5 or less to selectively precipitate uricase, but not more soluble contaminants. After washing at low pH (7-8) the uricase is then solubilized at a pH of about 10.2. The uricase preparation could then be analyzed by methods known in the art of pharmaceutical preparation, such as, for example, any one of high performance liquid chromatography (HPLC), other chromatographic methods, light scattering, centrifugation and/or gel electrophoresis.

Brief Description of the Drawings

Figure 1. SDS-mercaptoethanol PAGE (12% gel) analysis

Figure 2. Circulating life of native and PEGylated PBC uricase.

Figure 3. Relationship of serum uricase activity to the serum and urine concentrations of uric acid.

Figure 4. Maintenance of circulating level of uricase activity (measured in serum) after repeated injection.

Figure 5 shows the deduced amino acid sequences of pig-baboon chimeric uricase (PBC uricase) and porcine uricase containing the mutations R291K and T301S (PKS uricase), compared with the porcine and baboon sequences.

Figure 6. Comparison of amino acid sequences PKS and pig uricase.

Figure 7. Comparison of amino acid sequences of PBC and PKS.

Figure 8. Comparison of amino acid sequences of PBC and pig uricase.

Figure 9. Comparison of amino acid sequence of pig uricase and D3H.

Figure 10. Comparison of amino acid sequences of PBC and D3H.

Figure 11-1 and 11-2. Bestfit (GCG software) comparison of coding sequences of the cDNAs of PKS and pig uricase.

Figure 12-1 and 12-2. Bestfit (GCG software) comparison of coding sequences of the cDNAs of PKS and baboon uricase.

Figure 13-1 and 13-2. Bestfit (GCG software) comparison of coding sequences of the cDNAs of PBC and pig uricase.

Figure 14-1 and 14-2. Bestfit (GCG software) comparison of coding sequences of the cDNAs of PBC and baboon uricase.

Detailed Description of the Invention

The present invention provides uricase proteins which are useful intermediates for improved uricase conjugates of water-soluble polymers, preferably poly(ethylene glycols) or poly(ethylene oxides), with uricases. Uricase, as used herein, includes individual subunits as well as the native tetramer, unless otherwise indicated.

Although humans do not make an active enzyme, uricase mRNA transcripts have been amplified from human liver RNA (Wu et al. 1992). It is theoretically possible that some human uricase transcripts are translated; even if the peptide products were not full length or were unstable, they could be processed by antigen presenting cells and play a role in determining the immunologic response to an exogenous uricase used for treatment. It may, in theory, be possible to reconstruct and express a human uricase cDNA by eliminating the two known nonsense mutations. However, in the absence of selective pressure, it is very likely that deleterious missense mutations have accumulated in the human gene during the millions of years since the first nonsense mutation was introduced (Wu et al. 1989; Wu et al. 1992). Identifying and "correcting" all mutations to obtain maximal catalytic activity and protein stability would be very difficult.

The present inventors have appreciated that there is a high degree of homology (similarity) between the deduced amino acid sequence of human uricase to those of pig (about 86%) and baboon (about 92%) (see, Figures 6-14, for example of measure of similarity), whereas homology (similarity) between human and *A. flavus* uricase is <40% (Lee et al. 1988; Reddy et al. 1988; Wu et al. 1989; Legoux et al. 1992; Wu et al. 1992). The present invention provides recombinantly produced chimeric uricase proteins from two different mammals which have been designed to be less immunoreactive to humans than more distantly related fungal or bacterial enzyme. Use of a mammalian uricase derivative is expected to be more acceptable to patients and their physicians.

Experience has shown that activated PEGs such as have been used to make PEG-ADA and to modify other proteins attach via primary amino groups of the amino terminal residue (when present and unblocked) and epsilon-amino groups of lysines. This strategy is useful both because mild reaction conditions can be used, and because positively charged lysines tend to be located on the surfaces of proteins. The latter is important since for any therapeutic protein the desired effects of PEGylation will depend in part on the characteristics of the PEG polymer (e.g. mass, branched or unbranched structure, etc.) as well as on the number and distribution of PEG attachment sites of the protein relative to the epitopes and structural elements that determine function and clearance of the protein. A strategy for enhancing the ability of PEGylation to 'mask' epitopes and reduce immunogenicity by semi-selectively introducing novel lysine residues for potential PEG addition has been devised (Hershfield et al. 1991). This strategy employs mutagenesis to replace selected arginine codons with lysine codons, a substitution that maintains positive charge and has minimal effect on computer-predicted indices of surface probability and antigenicity (useful when only amino acid sequence is known).

As an experimental test of this strategy, recombinant *E. coli* purine nucleoside phosphorylase (EPNP) (Hershfield et al. 1991) has been used. Arg-to-Lys substitutions at 3 sites were introduced, increasing the number of lysines per subunit from 14 to 17, without altering catalytic activity. The purified triple-mutant retained full activity after modification of ~70% of accessible NH₂ groups with excess disuccinyl-PEG5000.

Titration of reactive amino groups before and after PEGylation suggested that the triple mutant could accept one more PEG strand per subunit than the wild type enzyme. PEGylation increased the circulating life of both the wild type and mutant EPNP enzymes in mice from ~4 hours to >6 days. After a series of intraperitoneal injections at weekly/biweekly intervals, all mice treated with both unmodified EPNPs, and 10 of 16 mice (60%) injected with PEGylated wild type EPNP, developed high levels of anti-EPNP antibody and a marked decline in circulating life. In contrast, only 2/12 mice (17%) treated with the mutant PEG-EPNP developed rapid clearance; low levels of antibody in these mice did not correlate with circulating life. This strategy was thus successful in substantially reducing immunogenicity even though only 1 of the 3 new lysines became modified after treatment with activated PEG.

The baboon and pig uricase subunits each consist of 304 amino acids, 29 of which (i.e. 1 in about 10 residues) are lysines. Initially attempts to introduce 2 Arg-to-Lys substitutions into the cloned cDNA for baboon uricase, and also a substitution of Lys for a Glu codon at position 208, which is known to be a Lys in the human uricase gene, resulted in an expressed mutant baboon protein which had greatly reduced uricase catalytic activity. It was apparent from this experiment that the ability to maintain uricase enzyme activity after arginine to lysine mutation of the mammalian DNA sequence was not predictable.

Subsequently, it was appreciated that amino acid residue 291 in the baboon uricase is lysine, but the corresponding residue in pig is arginine. The *Apal* restriction site present in both cDNAs was exploited to construct a chimeric uricase in which the first 225 amino acids are derived from the pig cDNA and the carboxy terminal 79 are derived from the baboon cDNA. The resulting pig-baboon chimeric (PBC) uricase (SEQ ID NO:2) possesses 30 lysines, one more than either "parental" enzyme. An additional feature of the PBC uricase is that its "baboon" portion differs from human uricase at 4 of 79 amino acid residues, whereas pig and human uricase differ at 10 in the same region. A modified version of PBC was subsequently constructed, which maintains the extra lysine at position 291 and otherwise differs from pig uricase only by a substitution of serine for threonine at residue 301 ("pigKS" uricase (SEQ ID NO:4)). In view of the results described in the preceding paragraph wherein several other insertions of lysines

were deleterious to activity, it was unexpected that the PBC and PKS chimeric uricase were fully as active as compared to the unmutated native pig uricase and approximately more than four fold active than unmutated native baboon uricase.

The present invention provides a recombinant pig-baboon chimeric uricase, composed of portions of the pig and baboon liver uricase sequences. One example of such a chimeric uricase contains the first 225 amino acids from the porcine uricase sequence (SEQ ID NO: 7) and the last 79 amino acids from the baboon uricase sequence (SEQ ID NO: 6) (pig-baboon uricase, or PBC uricase; Figure 6 and SEQ ID NO:2). Another example of such a chimeric uricase contains the first 288 amino acids from the porcine sequence (SEQ ID NO: 7) and the last 16 amino acids from the baboon sequence (SEQ ID NO: 6). Since the latter sequence differs from the porcine sequence at only two positions, having a lysine (K) in place of arginine at residue 291 and a serine (S) in place of threonine at residue 301, this mutant is referred to as pig-K-S or PKS uricase.

Vectors (expression and cloning) including the nucleic acid molecules coding the proteins of the present invention are also provided. Preferred vectors include those exemplified herein. One of ordinary skill will appreciate that nucleic acid molecules may be inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the nucleic acid (DNA) may be linked to appropriate transcriptional and translational regulatory nucleotide sequences recognized by the desired host, although such control elements are generally available in expression vectors used and known in the art. The vector may then be introduced into the host cells through standard techniques. Generally, not all of the host cells will be transformed by the vector. It may be necessary, therefore, to select transformed host cells. One such selection method known in the art involves incorporating into the expression vector a DNA sequence, with any necessary control elements, which codes for a selectable marker trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such a selectable trait may be in another vector which is used to co-transform the desired host cells. The vectors can also include an appropriate promoter, such as a prokaryotic promoter capable of expression (transcription and translation) of the DNA in a bacterial host cell, such as *E. coli*, transformed therewith.

Many expression systems are available and known in the art, including bacterial (for example *E. Coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

Suitable vectors may include a prokaryotic replicon, such as ColE1 *ori*, for propagation in, for example, a prokaryote. Typical prokaryotic vector plasmids are pUC18, pUC19, pUC322 and pBR329 available from Biorad Laboratories (Richmond, CA) and pTcr99A and pKK223-3 available from Pharmacia (Piscataway, NJ). A typical mammalian cell vector plasmid is pSVL available from Pharmacia (Piscataway, NJ). This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells. An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumor virus long terminal repeat to drive expression of the cloned gene. Useful yeast plasmid vectors are pRS403-406 and pRS413-416, and are generally available from Stratagene Cloning Systems (LaJolla, CA). Plasmids pRS403, pRS404, pRS405, and pRS406 are Yeast Integrating plasmids (Yips) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centomere plasmids (Ycps).

Moreover, the present invention provides host cells containing these vectors. Preferred host cells include those exemplified and described herein.

The uricase proteins of the present invention may be conjugated via a biologically stable, nontoxic, covalent linkage to a relatively small number of strands of PEG to improve the biological half-life and solubility of the proteins and reduce their immunoreactivity. Such linkages may include urethane (carbamate) linkages, secondary amine linkages, and amide linkages. Various activated PEGs suitable for such conjugation are commercially available from Shearwater Polymers, Huntsville, AL.

The invention also may be used to prepare pharmaceutical compositions of the uricase proteins as conjugates. These conjugates are substantially non-immunogenic and retain at least 70%, preferably 80%, and more preferably at least about 90% or more of the uricolytic activity of the unmodified enzyme. Water-soluble polymers suitable for use in the present invention include linear and branched poly(ethylene

glycols) or poly(ethylene oxides), all commonly known as PEGs. One example of branched PEG is the subject of U.S. Patent 5,643,575.

In one embodiment of the invention, the average number of lysines inserted per uricase subunit is between 1 and 10. In a preferred embodiment, the number of additional lysines per uricase subunit is between 2 and 8. It being understood that the number of additional lysines should not be so many as to be a detriment to the catalytic activity of the uricase. The PEG molecules of the conjugate are preferably conjugated through lysines of the uricase protein, more preferably, through a non-naturally occurring lysine or lysines which have been introduced into the portion of a designed protein which does not naturally contain a lysine at that position.

The present invention provides a method of increasing the available non-deleterious PEG attachment sites to a uricase protein wherein a native uricase protein is mutated in such a manner so as to introduce at least one lysine residue therein. Preferably, this method includes replacement of arginines with lysines.

PEG-uricase conjugates utilizing the present invention are useful for lowering the levels (i.e., reducing the amount) of uric acid in the blood and/or urine of mammals, preferably humans, and can thus be used for treatment of elevated uric acid levels associated with conditions including gout, tophi, renal insufficiency, organ transplantation and malignant disease.

PEG-uricase conjugates may be introduced into a mammal having excessive uric acid levels by any of a number of routes, including oral, by enema or suppository, intravenous, subcutaneous, intradermal, intramuscular and intraperitoneal routes. Patton, JS, et al., (1992) Adv Drug Delivery Rev 8:179-228.

The effective dose of PEG-uricase will depend on the level of uric acid and the size of the individual. In one embodiment of this aspect of the invention, PEG-uricase is administered in a pharmaceutically acceptable excipient or diluent in an amount ranging from 10 μ g to about 1 g. In a preferred embodiment, the amount administered is between about 100 μ g and 500 mg. More preferably, the conjugated uricase is administered in an amount between 1 mg and 100 mg, such as, for example, 5 mg, 20 mg, or 50 mg. Masses given for dosage amounts of the embodiments refer to the amount of protein in the conjugate.

Pharmaceutical formulations containing PEG-uricase can be prepared by conventional techniques, *e.g.*, as described in Remington's Pharmaceutical Sciences, (1985) Easton, PA: Mack Publishing Co. Suitable excipients for the preparation of injectable solutions include, for example, phosphate buffered saline, lactated Ringer's solution, water, polyols and glycerol. Pharmaceutical compositions for parenteral injection comprise pharmaceutically acceptable sterile aqueous or non-aqueous liquids, dispersions, suspensions, or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. These formulations can contain additional components, such as, for example, preservatives, solubilizers, stabilizers, wetting agents, emulsifiers, buffers, antioxidants and diluents.

PEG-uricase may also be provided as controlled release compositions for implantation into an individual to continually control elevated uric acid levels in blood and urine. For example, polylactic acid, polyglycolic acid, regenerated collagen, poly-L-lysine, sodium alginate, gellan gum, chitosan, agarose, multilamellar liposomes and many other conventional depot formulations comprise bioerodible or biodegradable materials that can be formulated with biologically active compositions. These materials, when implanted or injected, gradually break down and release the active material to the surrounding tissue. For example, one method of encapsulating PEG-uricase comprises the method disclosed in U.S. Patent No. 5,653,974, which is hereby incorporated by reference. The use of bioerodible, biodegradable and other depot formulations is expressly contemplated in the present invention. The use of infusion pumps and matrix entrapment systems for delivery of PEG-uricase is also within the scope of the present invention. PEG-uricase may also advantageously be enclosed in micelles or liposomes. Liposome encapsulation technology is well known in the art. See, *e.g.*, Lasic, D, et al., (Eds.) (1995) Stealth Liposomes, Boca Raton, FL: CRC Press.

The PEG-uricase pharmaceutical compositions described herein will decrease the need for hemodialysis in patients at high risk of urate-induced renal failure, *e.g.*, organ transplant recipients (see Venkatesh, VS, et al., (1990) Nephron 56:317-321) and patients with some malignant diseases. In patients with large accumulations of crystalline urate (tophi), such pharmaceutical compositions will improve the quality of life more rapidly than currently available treatments.

The following examples, which are not to be construed as limiting the invention in any way, illustrate the various aspects disclosed above.

EXAMPLE 1

5 A. Construction of PBC, PKS and related uricase cDNAs.

Standard methods, and where applicable instructions supplied by the manufacturers of reagents, were used for preparing total cellular RNA, for PCR amplification (U.S. Patent Nos. 4,683,195 and 4,683,202, 4,965,188 & 5,075,216) of
 10 urate oxidase cDNAs, and for cloning and sequencing of these cDNAs (Erlich 1989; Sambrook et al. 1989; Ausubel 1998). PCR primers for pig and baboon urate oxidases (Table 1) were designed based on published coding sequences (Wu et al. 1989) and using the PRIME software program (Genetics Computer Group, Inc.).

15 **Table 1. Primers for PCR Amplification of Urate Oxidase cDNA**

<u>Pig liver uricase cDNA:</u>	
sense:	5' gcgcgaattccATGGCTCATTACCGTAATGACTACA 3'.
Antisense:	5' gcgctctagaagcttccatggTCACAGCCTTGAAGTCAGC 3'.
<u>D3H baboon liver uricase cDNA:</u>	
sense:	5' gcgcgaattccATGGCCCACTACCATAACAACACTAT 3'
antisense:	5' gcgcccattggtctagaTCACAGTCTTGAAGACAACCTTCCT

Restriction enzyme sequences (lowercase) introduced at the ends of the primers are sense (pig and baboon) EcoRI and NcoI; antisense (pig) NcoI, HindIII, XbaI;
 20 antisense (baboon) NcoI. In the case of baboon sense primer, the third codon GAC (Aspartate) present in baboon urate oxidase (Wu et al. 1992) was replaced with CAC (Histidine), the codon that is present at this position in the coding sequence of the human urate oxidase pseudogene (Wu et al. 1992). For this reason the recombinant

baboon urate oxidase generated from the use of these primers has been named D3H baboon urate oxidase.

Total cellular RNA from pig and baboon livers was reverse-transcribed using a 1st strand kit (Pharmacia Biotech Inc. Piscataway, NJ). PCR amplification using Taq DNA polymerase (GibcoBRL, Life Technologies, Gaithersburg, MD) was performed in a thermal cycler (Ericomp, San Diego, CA) with the program [30 s, 95°C; 30 s, 55°C; 60 s, 70°C], 20 cycles, followed by [30 s, 95°C; 60 s, 70°C] 10 cycles. The urate oxidase PCR products were digested with EcoRI and HindIII and cloned into pUC18 (pig), and were also cloned directly (pig and D3H baboon) using the TA cloning system (Invitrogen, Carlsbad, CA). cDNA clones were transformed into the *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA). Plasmid DNA containing cloned uricase cDNAs was prepared and the cDNA insert sequence was analyzed by standard dideoxy technique. Clones that possessed the published urate oxidase DNA coding sequences (except for the D3H substitution in baboon urate oxidase described in Table I) were constructed and verified in a series of subsequent steps by standard recombinant DNA methodology.

The pig and D3H baboon cDNAs containing full length coding sequences were introduced into pET expression vectors (Novagen, Madison, WI) as follows. The D3H baboon uricase cDNA was excised from the TA plasmid with the NcoI and BamHI restriction enzymes and then subcloned into the NcoI and BamHI cloning sites of the expression plasmids pET3d and pET9d. Full length pig uricase cDNA was excised from a pUC plasmid clone with the EcoRI and HindIII restriction enzymes and subcloned into the EcoRI and HindIII sites of pET28b. The pig cDNA coding region was also introduced into the NcoI and BlnI sites of the expression plasmid pET9d after excision from the NcoI and BlnI sites of pET28b.

The pig-baboon chimera (PBC) cDNA was constructed by excising the 624 bp NcoI-ApaI restriction fragment of D3H baboon uricase cDNA from a pET3d-D3H-baboon clone; and then replacing this D3H baboon segment with the corresponding 624 bp NcoI-ApaI restriction fragment of pig cDNA. The resulting PBC urate oxidase cDNA consists of the pig urate oxidase codons 1-225 joined in-frame to codons 226-304 of baboon urate oxidase.

The pig-KS urate oxidase (PigKS) cDNA was constructed by excising the 864 bp NcoI-NdeI restriction fragment of D3H baboon uricase cDNA from a pET3d-D3H baboon clone, and then replacing this D3H baboon segment with the corresponding 864 bp NcoI-NdeI restriction fragment of pig cDNA. The resulting PKS urate oxidase cDNA consists of the pig urate oxidase codons 1-288 joined in-frame to codons 289-304 of baboon urate oxidase.

The amino acid sequences of the D3H baboon, pig, PBC, and PKS urate oxidases are shown in Figure 5 and the SEQUENCE LISTING). Standard techniques were used to prepare 15% glycerol stocks of each of these transformants, and these were stored at -70°C. When each of these species was expressed and the recombinant enzymes isolated (Table 2), the pig, PBC chimera, and PigKS uricases had very similar specific activity, which was approximately 4-5 fold higher than the specific activity of recombinant baboon uricase. This order was confirmed in several other experiments. The specific activity of PBC uricase prepared by several different procedures varied over a 2-2.5-fold range.

Table 2:

Comparison of Expressed Recombinant Mammalian Uricases

Construct	Specific Activity*	Relative Activity
	(Units/mg)	(Chimera=1)
PBC	7.02	1.00
PigKS	7.17	1.02
Pig	5.57	0.79
Baboon	1.36	0.19

* Protein was determined by the Lowry method. Uricase activity was determined spectrophotometrically (Priest and Pitts 1972). The assay was carried out at 23-25°C in a 1 cm quartz cuvette containing a 1 ml reaction mixture (0.1 M sodium borate, pH 8.6, 0.1 mM uric acid). Uric acid disappearance was monitored by decrease in absorbance at

292 nm. One international unit (IU) of uricase catalyzes the disappearance of one μmol of uric acid per minute.

E. coli BL21(DE3)pLysS transformants of the 4 uricase cDNA-pET constructs indicated in Table 2 were plated on LB agar containing selective antibiotics (carbenicillin and chloramphenicol for pET3d (pigKS); kanamycin and chloramphenicol for pET9d (PBC, pig, baboon)), as directed in the pET System Manual (Novagen, Madison WI). 5-ml cultures (LB plus antibiotics) were inoculated with single transformant colonies and grown for 3 hours at 37°C. Then 0.1 ml aliquots were transferred to 100 ml of LB medium containing selective antibiotics and 0.1% lactose (to induce uricase expression). After overnight growth at 37°, bacterial cells from 0.5 ml aliquots of the cultures were extracted into SDS-PAGE loading buffer, and analyzed by SDS-mercaptoethanol PAGE; this established that comparable levels of uricase protein had been expressed in each of the 4 cultures (results not shown). The remaining cells from each 100 ml culture were harvested by centrifugation and washed in PBS. The cells were then re-suspended in 25 ml of phosphate-buffered saline, pH 7.4 (PBS) containing 1 mM AEBSF protease inhibitor (Calbiochem, San Diego, CA) and then lysed on ice in a Bacterial Cell Disruptor (Microfluidics, Boston MA). The insoluble material (including uricase) was pelleted by centrifugation (20,190 x g, 4°, 15 min). The pellets were washed twice with 10 ml of PBS, and then were extracted overnight at 4° with 2 ml of 1 M Na₂CO₃, pH 10.2. The extracts were diluted to 10 ml with water and then centrifuged (20,190 x g, 4°, 15 min). Uricase activity and protein concentrations were then determined.

EXAMPLE 2

Expression and isolation of recombinant PBC uricase (4 liter fermentor prep).

The pET3d-PBC uricase transformant was plated from a glycerol stock onto an LB agar plate containing carbenicillin and chloramphenicol, as directed in the Novagen pET System Manual. A 200 ml inoculum started from a single colony was prepared in

LB-antibiotic liquid medium on a rotary shaker (250 rpm) at 37°, using procedures recommended in the pET System Manual to maximize pET plasmid retention. At an OD₅₂₅ of 2.4, cells from this 200 ml culture were collected by centrifugation and resuspended in 50 ml of fresh medium. This suspension was transferred to a high density fermentor containing 4 liters of carbenicillin- and chloramphenicol-containing SLBH medium (the composition of SLBH medium, and the design and operation of the fermentor are described in (Sadler et al. 1974)). After 20 hours of growth under O₂ at 32° (OD₅₂₅ = 19) isopropylthiogalactoside (IPTG) was added to 0.4 mM to induce uricase production. After 6 more hours (OD₅₂₅ = 37) bacterial cells were harvested by centrifugation (10,410 x g, 10 min, 4°C), washed once with PBS, and stored frozen at -20°C.

The bacterial cells (189 g) were resuspended in 200 ml PBS and lysed while cooled in an ice/salt bath by sonication (Heat Systems Sonicator XL, probe model CL, Farmingdale, NY) for 4 x 40 second bursts at 100% intensity, with a 1 minute rest between bursts. PBS-insoluble material (which includes uricase) was pelleted by centrifugation (10,410 x g, 10 min, 4°C), and was then washed 5 times with 200 ml PBS. Uricase in the PBS-insoluble pellet was extracted into 80 ml of 1 M Na₂CO₃, pH 10.2 containing 1 mM phenylmethylsulfonylfluoride (PMSF) and 130 µg/ml aprotinin. Insoluble debris was removed by centrifugation (20,190 x g, 2 hours, 4°C). All further steps in purification were at 4°C (results summarized in Table 3).

The pH 10.2 extract was diluted to 1800 ml with 1 mM PMSF (to reduce Na₂CO₃ to 0.075 M). This was applied to a column (2.6 x 9 cm) of fresh Q-Sepharose (Pharmacia Biotech, Inc., Piscataway, NJ), which had been equilibrated with 0.075 M Na₂CO₃, pH 10.2. After loading, the column was washed successively with 1) 0.075 M Na₂CO₃, pH 10.2 until A₂₈₀ absorbance of the effluent reached background; 2) 10 mM NaHCO₃, pH 8.5 until the effluent pH fell to 8.5; 3) 50 ml of 10 mM NaHCO₃, pH 8.5, 0.15 M NaCl; 4) a 100-ml gradient of 0.15 M to 1.5 M NaCl in 10 mM NaHCO₃, pH 8.5; 5) 150 ml of 10 mM NaHCO₃ pH 8.5, 1.5 M NaCl; 6) 10 mM NaHCO₃ pH 8.5; 7) 0.1 M Na₂CO₃, pH 11 until the effluent pH was raised to 11. Finally, uricase was eluted with a 500 ml gradient from 0 to 0.6 M NaCl in 0.1 M Na₂CO₃, pH 11. The activity eluted in two A₂₈₀-absorbing peaks, which were pooled separately (Fraction A and

Fraction B, Table 3). Uricase in each of these pools was then precipitated by lowering the pH to 7.1 by slow addition of 1 M acetic acid, followed by centrifugation (7,000 x g, 10 min). The resulting pellets were dissolved in 50 ml of 1 M Na₂CO₃, pH 10.2 and stored at 4°C.

5

Table 3

Recombinant Pig-Baboon Chimeric (PBC) Uricase Purification

IPTG-induced Cell Paste = 189.6 g

Fraction	Total Protein mg	Uricase activity U/ml	Total Uricase Units	Specific Activity U/mg
pH 7 Sonicate + pH 7 Wash			74.9	
pH 10.2 Extract	4712	82.7	11,170	2.4
Q-Sepharose fraction A	820	11.5	1,081*	1.9
fraction B	1809	31.7	4,080	2.3
pH 7.1 precipitated & redissolved				
fraction A	598	35.0	1,748	3.0
fraction B	1586	75.5	3,773	2.4
Total Recovery	2184		5,521	

10 *The uricase present in fraction A began to precipitate spontaneously after elution from the column. Therefore activity measured at this stage of purification was underestimated.

EXAMPLE 3

Small scale preparation and PEGylation of recombinant PBC uricase.

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This example shows that purified recombinant PBC uricase can be used to produce a PEGylated uricase. In this reaction, all uricase subunits were modified (Figure 1, lane 7), with retention of about 60% of catalytic activity (Table 4).

10 A. Small scale expression and isolation of PBC uricase (Table 4, Figure 1).

A 4-liter culture of *E.coli* BL21(DE3)pLysS transformed with pET3d-PBC cDNA was incubated on a rotary shaker (250 rpm) at 37°. At 0.7 OD₅₂₅, the culture was induced with IPTG (0.4 mM, 6 hours). The cells were harvested and frozen at -20°C.

15 The cells (15.3 g) were disrupted by freezing and thawing, and extracted with 1 M Na₂CO₃, pH 10.2, 1 mM PMSF. After centrifugation (12,000 x g, 10 min, 4°C) the supernatant (85 ml) was diluted 1:10 with water and then chromatographed on Q-Sepharose in a manner similar to that described in Example 1. Pooled uricase activity from this step was concentrated by pressure ultrafiltration using a PM30 membrane
20 (Amicon, Beverly, MA). The concentrate was chromatographed on a column (2.5 x 100 cm) of Sephacryl S-200 (Pharmacia Biotech, Piscataway, NJ) that was equilibrated and run in 0.1 M Na₂CO₃, pH 10.2. Fractions containing uricase activity were pooled and concentrated by pressure ultrafiltration, as above.

25 B. PEGylation.

100 mg of concentrated Sephacryl S-200 PBC uricase (5 mg/ml, 2.9 µmol enzyme; 84.1 µmol lysine) in 0.1 M Na₂CO₃, pH 10.2 was allowed to react with a 2-fold excess (mol of PEG:mol uricase lysines) of an activated form of PEG at 4° for 60 min.

30 The PEGylated uricase was freed from any unreacted or hydrolyzed PEG by tangential flow diafiltration. In this step the reaction was diluted 1:10 in 0.1 M Na₂CO₃, pH 10.2

and diafiltered vs. 3.5 vol 0.1 M Na₂CO₃, pH 10.2, then vs. 3.5 vol 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.2. The filter-sterilized enzyme was stable at 4° for at least one month.

5 Table 4.

Summary of Purification and PEGylation of Recombinant Pig-Baboon Chimeric (PBC) Uricase

A. Purification Fraction	Total protein	Total uricase activity	Specific activity	Recovery of activity
	mg	μmol/min	μmol/min/mg	%
Crude extract	1565	1010	0.6	100
Q-Sepharose	355	1051	3.0	104
Sephacryl S-200	215	1170	5.5	116
B. PEGylation				
S-200 uricase	100	546	5.5	100
PEG-uricase	97	336	3.5	62

10

Figure 1 shows a SDS-mercaptoethanol PAGE (12% gel) analysis of fractions obtained during the purification and PEGylation of recombinant pig-baboon chimera (PBC) uricase. Lanes: 1= MW markers; 2= SDS extract of uninduced pET3d-PBC cDNA-transformed cells (*E. coli* BL21(DE3)pLysS); 3= SDS extract of IPTG-induced pET-PBC cDNA-transformed cells; 4= Crude extract (see Table 5); 5= concentrated Q-sepharose uricase pool; 6= concentrated Sephacryl S-200 uricase pool; 7= PEGylated Sephacryl S-200 recombinant PBC uricase.

The results shown in Table 4 show that the purified PBC uricase could be modified with retention of about 60% of catalytic activity. In this PEGylation reaction all of the uricase subunits were modified (Figure 1, lane 7). In studies not shown, the PEGylated enzyme had similar kinetic properties to unmodified PBC uricase (K_M 10-20

µM). Importantly, the modified enzyme was much more soluble than the unmodified enzyme at physiologic pH (>5 mg/ml in PBS vs. <1 mg/ml). The PEGylated enzyme could also be lyophilized and then reconstituted in PBS, pH 7.2, with minimal loss of activity. In other experiments, we compared the activities of this preparation of PEG-PBC uricase with the *A. flavus* uricase clinical preparation. At pH 8.6 in borate buffer, the *A. flavus* enzyme had 10-14 fold higher V_{max} and a 2 fold higher K_M. However, in PBS, pH 7.2, the PEG-PBC and unmodified fungal enzymes differed in uricase activity by <2 fold.

10 EXAMPLE 4

Circulating life in mice of unmodified and PEGylated PBC uricase.

Figure 2 shows the circulating life of native and PEGylated PBC uricase. Groups of mice (3 per time point) were injected IP with 1 unit of native (circles) or PEG-modified (squares) recombinant PBC uricase (preparation described in Example 3). At the indicated times, blood was obtained from sets of three mice for measuring serum uricase activity. The PEGylated uricase (described in Example 3) had a circulating half-life of about 48 hours, vs. <2 hours for the unmodified enzyme (Fig 2).

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EXAMPLE 5

Efficacy of PEGylated uricase of invention.

Figure 3 shows the relationship of serum uricase activity to the serum and urine concentrations of uric acid. In this experiment, a homozygous uricase-deficient knockout mouse (Wu et al. 1994) received two injections, at 0 and 72 hours, of 0.4 IU of recombinant PBC uricase that had been PEGylated. The uricase deficient knock-out mouse was used in this experiment because, unlike normal mice that have uricase, these knock-out mice, like humans, have high levels of uric acid in their blood and body

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fluids and excrete high levels of uric acid in their urine. These high levels of uric acid cause serious injury to the kidneys of these mice, which is often fatal (Wu et al. 1994).

The experiment shown in Figure 3 demonstrates that intraperitoneal injections of a PEGylated preparation of recombinant PBC uricase resulted in an increase in serum uricase activity, which was accompanied by marked decline in the serum and urinary concentrations of uric acid in a uricase-deficient mouse.

EXAMPLE 6

10 Nonimmunogenicity of construct-carrier complex

PEGylated recombinant PBC uricase was injected repeatedly into homozygous uricase-deficient mice without inducing accelerated clearance, consistent with absence of significant immunogenicity. This was confirmed by ELISA. Figure 4 shows maintenance of circulating levels of uricase activity (measured in serum) after repeated injection. PEGylated PBC uricase was administered by intraperitoneal injection at 6-10 day intervals. Serum uricase activity was determined 24 hours post injection.

EXAMPLE 7

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Covalent linkage to mutationally introduced lysine

PEGylation of purified recombinant PBC uricase should result in attachment of PEG to the novel lysine (residue 291). In this experiment a preparation of PBC uricase could be modified by PEGylation. It can be determined by means known in the art whether the peptide containing the novel lysine (residue 291) has been modified by PEGylation.

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- All documents cited above are incorporated herein, in their entirety, by
20 reference.

SEQUENCE LISTING

<110> HERSHFIELD, MICHAEL S.
KELLY, SUSAN J.

<120> URATE OXIDASE

<130> 1579-267

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gtc cga act ggc tat ggg aag gat atg ata aaa gtt ctc cat att cag	96
Val Arg Thr Gly Tyr Gly Lys Asp Met Ile Lys Val Leu His Ile Gln	
20 25 30	
cga gat gga aaa tat cac agc att aaa gag gtg gca act tca gtg caa	144
Arg Asp Gly Lys Tyr His Ser Ile Lys Glu Val Ala Thr Ser Val Gln	
35 40 45	
ctg act ttg agc tcc aaa aaa gat tac ctg cat gga gac aat tca gat	192
Leu Thr Leu Ser Ser Lys Lys Asp Tyr Leu His Gly Asp Asn Ser Asp	
50 55 60	
gtc atc cct aca gac acc atc aag aac aca gtt aat gtc ctg gcg aag	240
Val Ile Pro Thr Asp Thr Ile Lys Asn Thr Val Asn Val Leu Ala Lys	
65 70 75 80	
ttc aaa ggc atc aaa agc ata gaa act ttt gct gtg act atc tgt gag	288
Phe Lys Gly Ile Lys Ser Ile Glu Thr Phe Ala Val Thr Ile Cys Glu	
85 90 95	
cat ttc ctt tct tcc ttc aag cat gtc atc aga gct caa gtc tat gtg	336
His Phe Leu Ser Ser Phe Lys His Val Ile Arg Ala Gln Val Tyr Val	
100 105 110	
gaa gaa gtt cct tgg aag cgt ttt gaa aag aat gga gtt aag cat gtc	384
Glu Glu Val Pro Trp Lys Arg Phe Glu Lys Asn Gly Val Lys His Val	
115 120 125	
cat gca ttt att tat act cct act gga acg cac ttc tgt gag gtt gaa	432
His Ala Phe Ile Tyr Thr Pro Thr Gly Thr His Phe Cys Glu Val Glu	

1579-267

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Met Ala His Tyr Arg Asn Asp Tyr Lys Lys Asn Asp Glu Val Glu Phe

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Arg	Asp	Gly	Lys	Tyr	His	Ser	Ile	Lys	Glu	Val	Ala	Thr	Ser	Val	Gln	
		35					40				45					
Leu	Thr	Leu	Ser	Ser	Lys	Lys	Asp	Tyr	Leu	His	Gly	Asp	Asn	Ser	Asp	
	50					55					60					
Val	Ile	Pro	Thr	Asp	Thr	Ile	Lys	Asn	Thr	Val	Asn	Val	Leu	Ala	Lys	
	65				70					75					80	
Phe	Lys	Gly	Ile	Lys	Ser	Ile	Glu	Thr	Phe	Ala	Val	Thr	Ile	Cys	Glu	
				85					90					95		
His	Phe	Leu	Ser	Ser	Phe	Lys	His	Val	Ile	Arg	Ala	Gln	Val	Tyr	Val	
			100					105					110			
Glu	Glu	Val	Pro	Trp	Lys	Arg	Phe	Glu	Lys	Asn	Gly	Val	Lys	His	Val	
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His	Ala	Phe	Ile	Tyr	Thr	Pro	Thr	Gly	Thr	His	Phe	Cys	Glu	Val	Glu	
	130					135					140					
Gln	Ile	Arg	Asn	Gly	Pro	Pro	Val	Ile	His	Ser	Gly	Ile	Lys	Asp	Leu	
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Lys	Val	Leu	Lys	Thr	Thr	Gln	Ser	Gly	Phe	Glu	Gly	Phe	Ile	Lys	Asp	
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Gln	Phe	Thr	Thr	Leu	Pro	Glu	Val	Lys	Asp	Arg	Cys	Phe	Ala	Thr	Gln	
			180					185					190			
Val	Tyr	Cys	Lys	Trp	Arg	Tyr	His	Gln	Gly	Arg	Asp	Val	Asp	Phe	Glu	
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Ala	Thr	Trp	Asp	Thr	Val	Arg	Ser	Ile	Val	Leu	Gln	Lys	Phe	Ala	Gly	
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Gln Phe Thr Thr Leu Pro Glu Val Lys Asp Arg Cys Phe Ala Thr Gln
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 Val Tyr Cys Lys Trp Arg Tyr His Gln Cys Arg Asp Val Asp Phe Glu
 195 200 205
 Ala Thr Trp Gly Thr Ile Arg Asp Leu Val Leu Glu Lys Phe Ala Gly
 210 215 220
 Pro Tyr Asp Lys Gly Glu Tyr Ser Pro Ser Val Gln Lys Thr Leu Tyr
 225 230 235 240
 Asp Ile Gln Val Leu Ser Leu Ser Arg Val Pro Glu Ile Glu Asp Met
 245 250 255
 Glu Ile Ser Leu Pro Asn Ile His Tyr Phe Asn Ile Asp Met Ser Lys
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 35 40 45
 Leu Thr Leu Ser Ser Lys Lys Asp Tyr Leu His Gly Asp Asn Ser Asp
 50 55 60
 Val Ile Pro Thr Asp Thr Ile Lys Asn Thr Val Asn Val Leu Ala Lys
 65 70 75 80
 Phe Lys Gly Ile Lys Ser Ile Glu Thr Phe Ala Val Thr Ile Cys Glu
 85 90 95
 His Phe Leu Ser Ser Phe Lys His Val Ile Arg Ala Gln Val Tyr Val
 100 105 110
 Glu Glu Val Pro Trp Lys Arg Phe Glu Lys Asn Gly Val Lys His Val
 115 120 125
 His Ala Phe Ile Tyr Thr Pro Thr Gly Thr His Phe Cys Glu Val Glu
 130 135 140
 Gln Ile Arg Asn Gly Pro Pro Val Ile His Ser Gly Ile Lys Asp Leu
 145 150 155 160

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<220> Description of Artificial Sequence: RBC amino truncated

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Ser Ile Lys Glu Val Ala Thr Ser Val Gln Leu Thr Leu Ser Ser Lys
35 40 45
Lys Asp Tyr Leu His Gly Asp Asn Ser Asp Val Ile Pro Thr Asp Thr
50 55 60
Ile Lys Asn Thr Val Asn Val Leu Ala Lys Phe Lys Gly Ile Lys Ser
65 70 75
Ile Glu Thr Phe Ala Val Thr Ile Cys Glu His Phe Leu Ser Ser Phe
85 90 95
Lys His Val Ile Arg Ala Gln Val Tyr Val Glu Glu Val Pro Trp Lys
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Arg Phe Glu Lys Asn Gly Val Lys His Val His Ala Phe Ile Tyr Thr

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Arg Asp Gly Lys Tyr His Ser Ile Lys Glu Val Ala Thr Ser Val Gln
          35                               40                      45
Leu Thr Leu Ser Ser Lys Lys Asp Tyr Leu His Gly Asp Asn Ser Asp
      50                               55                      60
Val Ile Pro Thr Asp Thr Ile Lys Asn Thr Val Asn Val Leu Ala Lys
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Phe Lys Gly Ile Lys Ser Ile Glu Thr Phe Ala Val Thr Ile Cys Glu

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11
90

95

85
His Phe Leu Ser Ser Phe Lys His Val Ile Arg Ala Gln Val Tyr Val
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Glu Glu Val Pro Trp Lys Arg Phe Glu Lys Asn Gly Val Lys His Val
115 120 125
His Ala Phe Ile Tyr Thr Pro Thr Gly Thr His Phe Cys Glu Val Glu
130 135 140
Gln Ile Arg Asn Gly Pro Pro Val Ile His Ser Gly Ile Lys Asp Leu
145 150 155 160
Lys Val Leu Lys Thr Thr Gln Ser Gly Phe Glu Gly Phe Ile Lys Asp
165 170 175
Gln Phe Thr Thr Leu Pro Glu Val Lys Asp Arg Cys Phe Ala Thr Gln
180 185 190
Val Tyr Cys Lys Trp Arg Tyr His Gln Gly Arg Asp Val Asp Phe Glu
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Ala Thr Trp Asp Thr Val Arg Ser Ile Val Leu Gln Lys Phe Ala Gly
210 215 220
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225 230 235 240
Asp Ile Gln Val Leu Ser Leu Ser Arg Val Pro Glu Ile Glu Asp Met
245 250 255
Glu Ile Ser Leu Pro Asn Ile His Tyr Phe Asn Ile Asp Met Ser Lys
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35 40 45
Lys Asp Tyr Leu His Gly Asp Asn Ser Asp Val Ile Pro Thr Asp Thr

WO 00/08196

12

60

50
 Ile Lys Asn Thr Val Asn Val Leu Ala Lys Phe Lys Gly Ile Lys Ser
 65 70 75 80
 Ile Glu Thr Phe Ala Val Thr Ile Cys Glu His Phe Leu Ser Ser Phe
 85 90 95
 Lys His Val Ile Arg Ala Gln Val Tyr Val Glu Glu Val Pro Trp Lys
 100 105 110
 Arg Phe Glu Lys Asn Gly Val Lys His Val His Ala Phe Ile Tyr Thr
 115 120 125
 Pro Thr Gly Thr His Phe Cys Glu Val Glu Gln Ile Arg Asn Gly Pro
 130 135 140
 Pro Val Ile His Ser Gly Ile Lys Asp Leu Lys Val Leu Lys Thr Thr
 145 150 155 160
 Gln Ser Gly Phe Glu Gly Phe Ile Lys Asp Gln Phe Thr Thr Leu Pro
 165 170 175
 Glu Val Lys Asp Arg Cys Phe Ala Thr Gln Val Tyr Cys Lys Trp Arg
 180 185 190
 Tyr His Gln Gly Arg Asp Val Asp Phe Glu Ala Thr Trp Asp Thr Val
 195 200 205
 Arg Ser Ile Val Leu Gln Lys Phe Ala Gly Pro Tyr Asp Lys Gly Glu
 210 215 220
 Tyr Ser Pro Ser Val Gln Lys Thr Leu Tyr Asp Ile Gln Val Leu Thr
 225 230 235 240
 Leu Gly Gln Val Pro Glu Ile Glu Asp Met Glu Ile Ser Leu Pro Asn
 245 250 255
 Ile His Tyr Leu Asn Ile Asp Met Ser Lys Met Gly Leu Ile Asn Lys
 260 265 270
 Glu Glu Val Leu Leu Pro Leu Asp Asn Pro Tyr Gly Lys Ile Thr Gly
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 Thr Val Lys Arg Lys Leu Ser Ser Arg Leu
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<210> 11

<211> 301

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